Leptomyxid Ameba, a New Agent of Amebic Meningoencephalitis in Humans and Animals

G. S. VISVESVARA,[1]* A. J. MARTINEZ,[2] F. L. SCHUSTER,[3] G. J. LEITCH,[4] S. V. WALLACE,[1]
T. K. SAWYER,[5] AND M. ANDERSON[6]

*Centers for Disease Control, Atlanta, Georgia 30333[1]; School of Medicine, University of Pittsburgh, Pittsburgh,
Pennsylvania 15260[2]; Brooklyn College, City University of New York, New York, New York 10021[3]; Morehouse
School of Medicine, Atlanta, Georgia 30314[4]; RESCON Associates, Oxford, Maryland 21662[5]; and
San Diego Zoo, San Diego, California 92112[6]*

Amebae belonging to the order Leptomyxida are regarded as innocuous soil organisms incapable of infecting mammals. We report here the isolation of a leptomyxid ameba from the brain of a pregnant baboon (*Papio sphinx*) that died of meningoencephalitis at the San Diego Zoo Wild Animal Park. By using rabbit anti-leptomyxid serum in the immunofluorescence assay, we have identified the leptomyxid ameba in the brain sections of a number of human encephalitic cases from around the world as well as a few cases of meningoencephalitis in animals in the United States, which suggests that the leptomyxid amebae are potential etiologic agents of fatal meningoencephalitis in humans and animals.

Small, free-living amebae belonging to the genera *Acanthamoeba* and *Naegleria* are known to cause central nervous system (CNS) infections in humans (14). *Naegleria fowleri* causes an acute and fulminating infection, primary amebic meningoencephalitis, which almost always leads to death (7, 14, 25). In the United States only one patient has survived this disease (7, 14, 21, 25). Primary amebic meningoencephalitis occurs principally in children and young adults with a history of participation in swimming, diving, and other water sports in freshwater lakes and ponds. *Acanthamoeba* spp., on the other hand, cause infections of the human cornea as well as the CNS. The corneal infection, called *Acanthamoeba* keratitis, is a nonfatal, but nevertheless painful, vision-threatening disease and occurs primarily in contact lens wearers (23). CNS infection due to *Acanthamoeba* spp., called granulomatous amebic encephalitis (GAE), however, has occurred in immunosuppressed, chronic alcoholics and debilitated individuals who usually had no history of contact with fresh water (7, 12–15, 18, 25). Excepting a few (5, 10, 11), almost all cases of GAE have resulted in death. In addition to these, a few other cases of GAE thought to be due to some as-yet-unidentified ameba have also been described (3, 8, 9, 20). Currently the identification of the causative agent of GAE, in the absence of culture isolation, is on the basis of the presence of amebic trophozoites and cysts (cysts are not usually seen in primary amebic meningoencephalitis infections) in the CNS and/or reactivity of the amebae in tissue sections in the indirect immunofluorescence test (IIF) with the antiserum made against several species of *Acanthamoeba* (24). However, six cases (2, 4, 6, 16, 29; S. L. Nielson, presented at the Diagnostic Slide Session of the 60th Annual Meeting of the American Association of Neuropathologists, San Diego, Calif., 1984) of human infections (five from the United States and one from Australia) have been considered to be due to *Acanthamoeba* spp., even in the absence of clear-cut immunohistologic tests (Table 1). Additionally, specimens from several cases of GAE from around the world (four from the United States,

two from Peru, and one [each] from Canada, Mexico, Venezuela, and Argentina) have been submitted to the Centers for Disease Control for clinical diagnosis and identification (Table 1).

We present in this report a case of meningoencephalitis caused by a free-living ameba hitherto regarded as an innocuous soil organism incapable of infecting mammals. The index case, from the brain of which the ameba was isolated, involved a 3-year, 10-month-old pregnant mandrill (*Papio sphinx*) that died of meningoencephalitis at the San Diego Zoo Wild Animal Park.

(This work was presented in part by G. S. Visvesvara at the 5th International Conference on Biology and Pathogenicity of Free Living Amoebae, Brussels, Belgium, 7 to 11 August, 1989.)

MATERIALS AND METHODS

Case report. The previously healthy mandrill was observed to drag her right arm and appeared depressed. On examination, no fracture or swelling of limbs or joints was noticed. She was in the third trimester of pregnancy. Two days later she developed a daydreaming expression, appeared lethargic, and was found incapable of any movement. She died a few hours later. Postmortem cesarean section revealed a premature, hairless, dead infant. At the time of necropsy a small fragment of necrotic cerebral hemisphere was removed and frozen at −20°C, and the rest of the brain was fixed in 10% neutral-buffered Formalin. Microscopic examination of the fixed brain sections revealed amebic organisms. The frozen brain was sent to the Centers for Disease Control on dry ice. The fragment of cerebrum was minced in a small amount (ca. 0.5 ml) of sterile saline, inoculated on (i) a nonnutrient agar plate covered with a lawn of *Escherichia coli* and on (ii) a 3-day-old monolayer of monkey kidney cell (E6) culture and (iii) a culture of human embryonic-lung cell line (MRC-5) (26–28), and incubated at 37°C.

Mouse pathogenicity tests. A group of 10 6- to 8-week-old mice was instilled intranasally with 10 µl of saline containing about 1,000 trophozoites and cysts concentrated from E6 cell culture, fed ad libitum on micromixed chow, and in-

TABLE 1. Cases of GAE due to the leptomyxid ameba in humans and animals

Subject no. (reference or source)[a]	Age (yr)	Sex[b]	Location (yr)	Clinical course (days)	Clinical history and/or associated illness
Human					
1 (4)	2.5	F	Australia (1974)	30	Occipital headache 2 days prior to illness
2 (6)	0.3	M	South Carolina (1978)	37	Intermittent diarrhea, dehydration, high temperature, and bilateral pneumonia
3 (29)	2.5	M	Pennsylvania (1979)	240	6-mo history of right-sided neurologic dysfunction, headache, and vomiting
4 (Nielson[c])	72	M	California (1983)	14	Chronic renal failure, on hemodialysis and azotemia due to arterionephrosclerosis
5 (*)	0.75	F	California (1983)	15	Diarrhea, cough, high fever, and 6th-nerve palsy
6 (*)	61	M	Florida (1983)	30	Chronic alcoholic, deterioration of mental status, and healed scars on knee
7 (*)	22	M	Canada (1983)	72	Head injury, throbbing bitemporal headache, seizure, and craniotomy
8 (16)	11	F	Texas (1986)	180	Generalized seizures, headache, intermittent fever, and vomiting
9 (2)	36	M	New York (1988)	20	Skin abscess, intravenous drug abuse, and acquired immunodeficiency syndrome
10 (19)	39	M	Mexico (1988)	120	Grand mal seizure 4 mo before death, multiple cranial nerve deficit, and coma
11 (*)	7	M	Venezuela (1988)	210	Bilateral exophthalmus, Pyle's disease, and mild focal interstitial pneumonitis
12 (*)	22	M	Peru (1988)	150	5-mo history of acute headache, seizure, and nasal tumor
13 (*)	30	M	Peru (1988)	90	3-mo history of headache, nausea, vomiting, dizziness, and diplopia
14 (*)	12	M	Argentina (1989)	14	Lesions in the nares, upper lips, and arm
15 (*)	60	M	Georgia (1989)	120	Amputation of right leg at knee because of an accident and skin abscess
16 (*)	60	M	Nevada (1990)		Chronic alcoholic, seizures, and hemiparesis
Animal					
1 (1; gorilla)	1	F	California (1982)	14	Nonhealing sores on the head, lesions in the brain, weakness, and incoordination
2 (this study; mandrill)	3.83	F	California (1986)	14	See case history in Materials and Methods
3 (*; sheep)	Unknown	F	Texas (1989)	14	Pruritis on the head, incoordination and blindness, and unsteady gait

[a] *, Submitted to the Centers for Disease Control for identification.
[b] M, Male; F, female.
[c] Nielson, 60th AMAAN.

spected daily until they died or were killed by carbon dioxide inhalation. All experimental animals were housed and cared for in accordance with the guidelines and standards set by the Centers for Disease Control. Mice showing symptoms of meningoencephalitis were killed, and the brains were fixed in either 10% neutral buffered Formalin or Karnovsky's fixative (see below) or processed for culture on agar plates and E6 cells, as described above.

Scanning and transmission electron microscopy. About 5,000 trophozoites and cysts in 100 μl of Eagle minimum essential medium were inoculated onto the E6 monolayer. Two days later the medium from the monolayer flask was removed and the monolayer was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, for 2 h at 4°C in preparation for scanning electron microscopy. The samples were dehydrated through a graded series of ethanol, dried in a critical-point drier, and sputter coated with gold-palladium prior to being mounted and examined with a JEOL JSM 820 scanning electron microscope. Amebic trophozoites and cysts from 3- to 5-day-old E6 cell cultures were also harvested by centrifugation at 500 × g for 10 min, and the pellet was fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, for transmission electron microscopy. Fragments of the infected mouse brain tissue were fixed in Karnovsky's fixative at room temperature for transmission

electron microscopy. Both the brain tissue fragments and the culture pellets were postfixed in a solution of 1% OsO_4, dehydrated in ethanol, and embedded in Epon 812. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Philips 200 electron microscope.

Generation of antibody and IIF. Antibodies to the leptomyxid ameba as well as to several *Acanthamoeba* species (i.e., *A. castellanii, A. polyphaga, A. culbertsoni, A. rhysodes, A. palestinensis, A. astronyxis*) and to *N. fowleri, Naegleria gruberi, Hartmannella vermiformis, Vahlkampfia avara,* and *Entamoeba histolytica* were prepared in rabbits by multiple intravenous injections of washed trophozoites and cysts from culture (24). For example, trophozoites and cysts of the leptomyxid ameba from E6 cell culture were washed thrice with Hanks balanced salt solution by centrifugation at 250 × g and suspended in Hanks balanced salt solution to obtain 10^6 (with a 3:1 ratio of trophozoites to cysts) per ml. A total of 0.1 ml of this suspension was injected into the marginal ear vein of a rabbit on day 0, and the rest of the suspension was frozen at −20°C in 0.1-ml aliquots. The rabbit was boosted on days 21, 28, 35, and 49 with 0.1 ml of the quickly thawed suspension of the preparation that was frozen on day 0. The rabbit received on day 74 a final booster which consisted of 0.1 ml of 2 × 10^5 amebae (with a 4:1 ratio of trophozoites to cysts) obtained
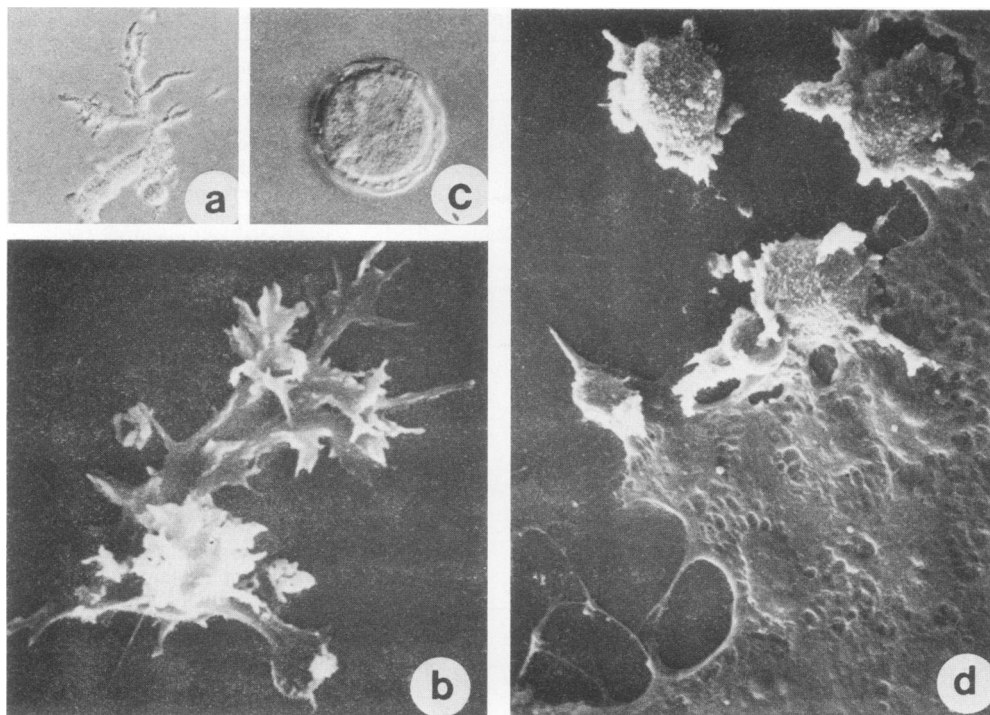
FIG. 1. (a) Phase-contrast (magnification, ×600) and (b) scanning electron (magnification, ×1,500) micrographs of a trophozoite. Note the irregular shape and extensive branching. (c) Typical cyst. Magnification, ×800. Note the wavy outer cyst wall. (d) Monolayer of monkey kidney cells partially destroyed by the amebae.

from fresh cultures washed as described above. The rabbit was bled on day 84, and the serum was separated, aliquoted into 1-ml volumes, and stored at −20°C until used. IIF was performed as described previously (24) on trophozoites and cysts obtained from E6 cultures and on paraffin-embedded brain sections of infected mouse, baboon, gorilla, sheep, and humans (Table 1).

RESULTS

Agar plates inoculated with the minced baboon brain tissue did not show any growth of amebae even after 4 weeks of incubation at 37°C. The MRC-5 cell line (after 3 weeks of incubation) also did not show any amebic growth but became heavily contaminated with fungi and was therefore discarded. The control cell cultures inoculated with sterile saline did not show any growth of amebae. Amebic growth was seen only in the E6 cell line after 3 weeks of incubation. The amebae were therefore passaged on the E6 cell line, initially once in 4 to 6 weeks and thereafter once a week. Two to three days after inoculation of the E6 cell line with the amebae, transformation of trophozoites to cysts began to occur. At the end of a week, equal numbers of amebic trophozoites and cysts were observed, as was the total destruction of cell sheets. After six to eight consecutive monthly transfers, the amebae regularly destroyed the cell monolayer within a week. Characteristic cytopathic effects (26, 27) such as vacuolization, nuclear pycnosis, and discontinuity of the cell sheet eventually led to total destruction of the cell sheet within 5 to 7 days.

The trophic amebae were mostly irregular in shape, were sometimes highly branched, and measured 15 to 60 μm (Fig. 1a and b). Occasionally a few elongated forms measuring 60

to 120 μm in length and 15 to 20 μm in breadth with several contractile vacuoles were also seen. The amebae were uninucleate, but a few binucleate forms were also seen. The binucleate forms may have completed karyokinesis but not cytokinesis. The nucleus, about 5 μm in diameter, had a centrally located nucleolus. No cytoplasmic streaming was noted. Cysts (Fig. 1c) were mostly uninucleate with occasional binucleate forms. The cysts were irregularly round, measuring about 15 to 30 μm with two walls. The inner cyst wall was thin and spherical. The outer cyst wall, however, was thick, wavy, and irregular.

All mice inoculated with the leptomyxid amebae showed symptoms of the disease (e.g., ruffled fur, aimless wandering in circles, partial paralysis, and coma) and died within a week. None of the agar plates inoculated with the mouse brain tissues showed any growth of the amebae. However, the amebae grew readily on the E6 monolayer within 1 to 2 weeks of incubation and destroyed the cell sheet, as described above.

Microscopic examination of the CNS sections of the mandrill revealed profuse, necrotizing meningoencephalitis with numerous amebic trophozoites and a few cysts (Fig. 2a and b). In the mandrill, clusters of trophozoites were seen around blood vessels and were accompanied by mononuclear cells. The amebae around the blood vessels and elsewhere in the brain tissue stained brightly when reacted with the rabbit anti-leptomyxid serum in the IIF (Fig. 2c and d). In other areas diffuse, chronic inflammatory infiltration with sparse multinucleated giant cells and diffuse lymphocytic and plasma cells was seen. Some intraparenchymal blood vessels were surrounded by numerous lymphocytes, plasma cells, and macrophages. Scattered foci of polymorphonuclear leukocytes were also seen within the most affected
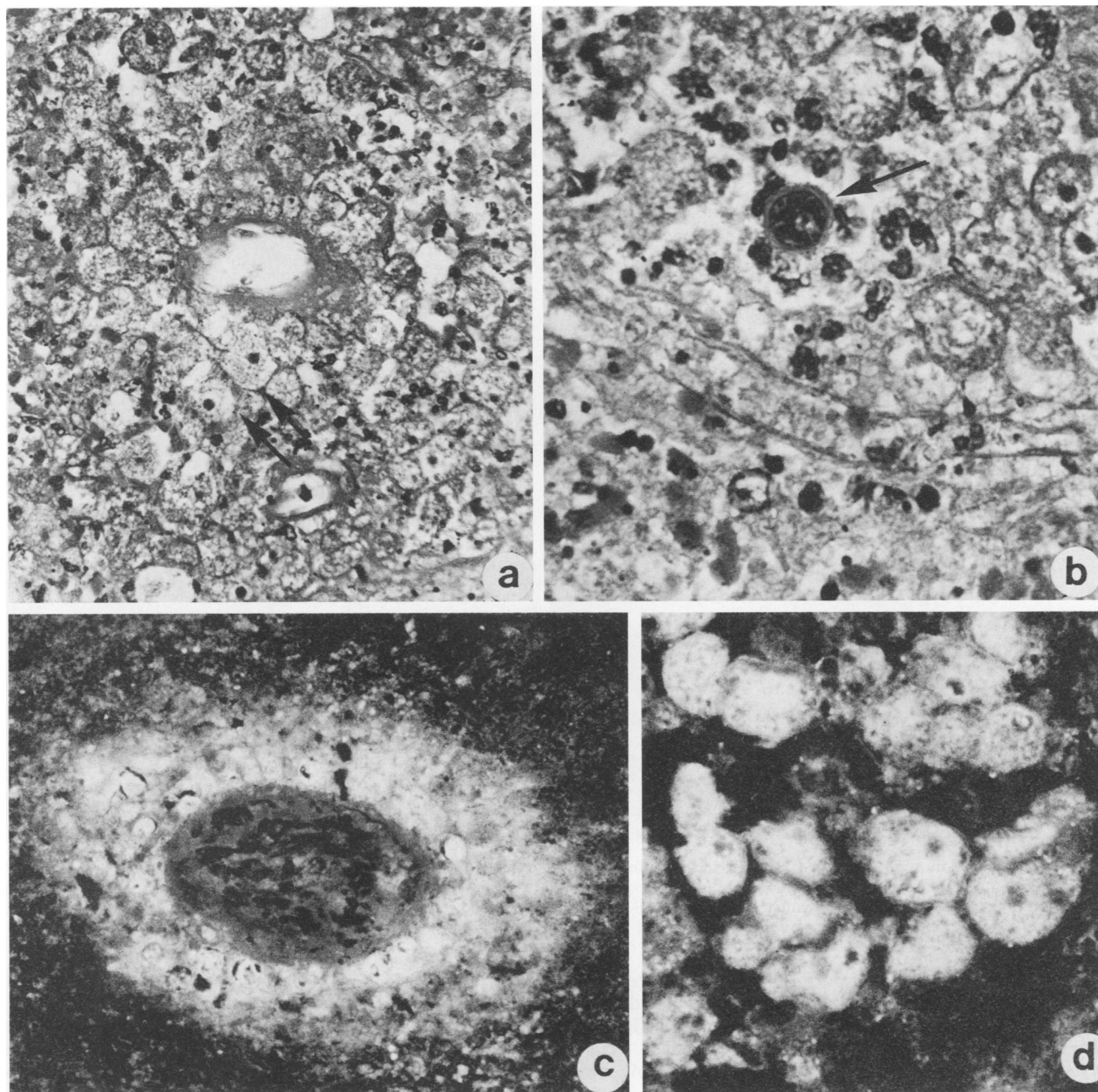
FIG. 2. (a) Mandrill brain showing a blood vessel encompassed by numerous amebic trophozoites (at arrows) (stained with hematoxylin and eosin). Magnification ×400. (b) Cyst (arrow) of leptomyxid ameba around a perivascular space in the mandrill brain. Magnification, ×550. Note the dense cyst wall (hematoxylin and eosin). (c and d) Immunofluorescence staining of leptomyxid amebae around a blood vessel (c; magnification, ×100) and elsewhere (d; magnification, ×400) in the mandrill brain. The brain sections were first reacted with the rabbit anti-leptomyxid ameba serum and subsequently reacted with goat anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate.

areas. The CNS lesions in the mice, while similar to those of the mandrill, were less pronounced. Ultrastructurally the amebic trophozoites were characterized by a centrally placed, dense nucleolus surrounded by a clear nuclear halo and abundant granular cytoplasm. Intracytoplasmic organelles were abundant and consisted of lysosomes, mitochondria, Golgi apparatus, myelin figures, rough and smooth endoplasmic reticula, lipid droplets, and empty vacuoles. Ribosomes were also detected around rough endoplasmic reticula. Dense, membrane-bound bodies were abundant. The nucleus was round and was enclosed by a double membrane (Fig. 3a). The nucleolus was compact. In many trophozoites, two or more nucleoli were seen. The mitochondria, irregularly round and oval, were numerous and scattered throughout the cytoplasm. The intramitochondrial cristae were irregular and had a honeycomb pattern (Fig. 3b). The cysts were round with a compound wall consisting of three layers. The inner wall (endocyst) was dense and uniform and lined by a thin, wavy layer. The ectocyst, or outer wall, was thinner and irregular, with protrusions.

An amorphous third layer (mesocyst) was present between the ectocyst and endocyst. Some of the cysts showed
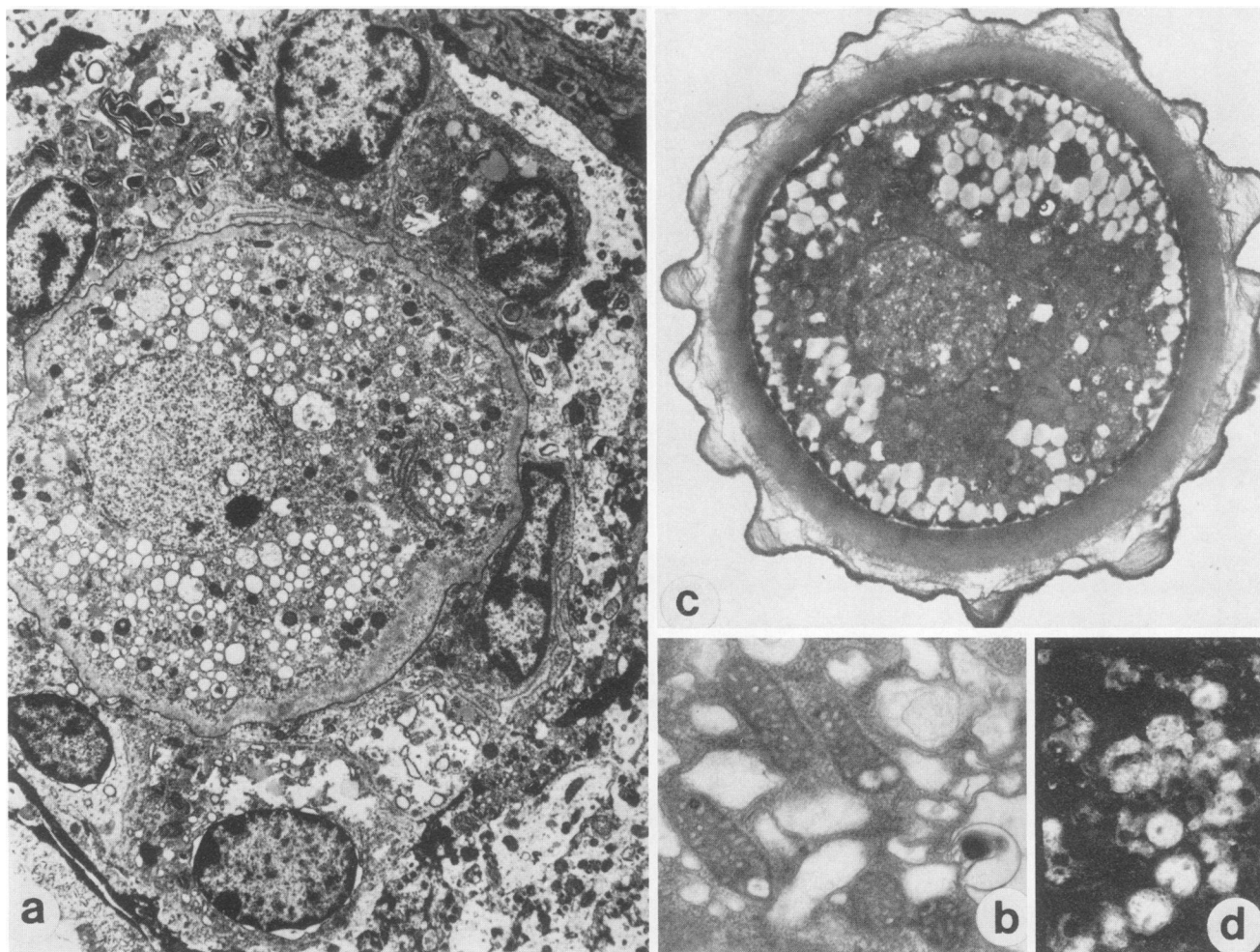
FIG. 3. (a) Electron micrograph of CNS of a mouse infected intranasally with the leptomyxid ameba. Note the activated microglia or macrophagelike cells surrounding the ameba. Magnification, ×4,000. (b) Details of mitochondria. Magnification, ×25,000. (c) Electron micrograph of a triple-walled cyst showing a thick inner wall, thin outer layer, and reticulate material in between. Magnification, ×7,500. (d) Immunofluorescence patterns of amebae within the CNS of a mouse infected with the leptomyxid ameba. Magnification, ×100.

a stellate pattern. In the inner portion of the cyst there were seen compactly arranged mitochondria, granular and electron-dense bodies, and numerous lipidlike droplets (Fig. 3c).

In the IIF none of the antisera made against *Acanthamoeba* spp. (i.e., *A. castellanii*, *A. polyphaga*, *A. rhysodes*, *A. culbertsoni*, *A. astronyxis*, *A. palestinensis*), *N. fowleri*, *N. gruberi*, *H. vermiformis*, *V. avara*, and *Entamoeba histolytica* reacted with the amebae in the brain sections of the mandrill, gorilla, sheep, or the various human cases listed in Table 1 or the sections of the brain of the mice inoculated experimentally with the leptomyxid amebae, even at a 1:50 dilution of the antisera. In the homologous reactions the various sera reacted with the homologous antigens (amebae) at a dilution of 1:128 or higher (*A. castellanii*, 512; *A. polyphaga*, 1,024; *A. rhysodes*, 256; *A. culbertsoni*, 1,024; *A. astronyxis*, 256; *A. palestinensis*, 256; *N. fowleri*, 2,056; *N. gruberi*, 1,024; *H. vermiformis*, 128; *V. avara*, 128; *Entamoeba histolytica*, 256; and leptomyxid ameba, 512). The anti-leptomyxid serum, however, reacted extensively with the leptomyxid amebae from culture as well as those in the brain sections at a dilution of 1:50 and moderately at a dilution of 1:100 and produced the characteristic bright apple green fluorescence (Fig. 2c and d and

3d). The anti-leptomyxid serum did not react with the heterologous amebae at these dilutions. These tests clearly indicate that the cases listed in Table 1 were caused by the leptomyxid ameba rather than *Acanthamoeba* spp. or other amebae. Furthermore, when the anti-leptomyxid ameba serum absorbed with the leptomyxid ameba grown in culture was used, no fluorescence was seen, whereas no appreciable decrease in the fluorescence was noticed when the anti-leptomyxid serum absorbed with *Acanthamoeba* spp., *Naegleria* spp., *H. vermiformis*, *V. avara*, or *Entamoeba histolytica*. The cross absorption experiments further confirm the above studies and indicate that the amebae in these tissues are antigenically similar to the leptomyxid ameba.

DISCUSSION

Among the hundreds of free-living amebae that exist in nature, amebae belonging to only two genera (*Acanthamoeba* and *Naegleria*) are known to cause disease in humans. Although some species of *Hartmannella*, *Vahlkampfia*, *Paramoeba*, *Vexellifera*, and *Nuclearia* have been described in the literature as the causal agents of disease in invertebrates such as snails, oysters, crabs, fish,

and the parasitic helminth *Schistosoma*, no true hartmannellid ameba is known to cause disease in humans or other mammals (28a). In the earlier literature, because of taxonomic and nomenclatural difficulties, references to *Hartmannella* spp. or hartmennellid amebae as agents of human disease exist. Since no true *Hartmannella* spp. have been known to cause infection in mammals until the present, these should be corrected to read as *Acanthamoeba* spp. Additionally, reports of two cases of encephalitis probably due to *Vahlkampfia* spp. exist in the literature. In these cases identification was made on the basis of the presence of double nucleoli in the nuclei of the amebae present in the tissue even in the absence of serologic evidence (7). The cases reported above probably were caused by the leptomyxid ameba rather than *Vahlkampfia* spp.

It is interesting to note that most of the free-living amebae that we are familiar with (e.g., the genera *Acanthamoeba*, *Naegleria*, *Hartmannella*, and *Vahlkampfia*) can be grown in association with either *E. coli* or *Enterobacter aerogenes* on agar plates. The leptomyxid ameba reported here, however, failed to grow repeatedly on agar with the bacterial overlay. It is also interesting that attempts to isolate the amebae from some of the human GAE cases (4, 7, 14, 29) have failed, which clearly indicates that not all amebae will grow on agar. It is quite likely that some of these amebae probably have very particular nutritional requirements and other culture conditions (such as temperature of incubation) that are unknown to us at the present time. It is therefore suggested that in future isolation attempts from GAE cases, efforts should be made to inoculate mammalian cell cultures in addition to nonnutrient agar plates layered with bacteria. The amebae in the present case grew on monkey kidney cells and probably would have grown on the MRC cell line as well had it not been for the heavy contamination with fungi. We have, since then, been able to grow these amebae on the MRC and HEp-2 as well as diploid macrophage cell lines. It is also possible that the trophozoites in the brain tissue were destroyed because the tissue was frozen and that the cysts which survived the freezing later destroyed the monolayer. This may be one of the reasons it took so long for the ameba to establish in culture.

Amebae belonging to the order Leptomyxida, Pussard and Pons 1976 (17), are relatively uncommon and are generally considered innocuous soil organisms. To date only five genera comprising five to seven species have been included in this order. With the exception of *Paraflabellula* (*Flabellula*) *kudoi* Singh and Hanumaiah 1979 (17, 22), which has been shown to grow at higher temperatures (42°C), none of the other amebae included in this order have been reported to grow at 37°C. Our incomplete knowledge concerning human infections with the leptomyxid amebae coupled with the recent reports of such human infections thought to be due to *Acanthamoeba* spp., even in the absence of clear-cut immunohistologic tests (4, 6, 16, 29; Nielson, 60th AMAAN), suggests that one should exercise caution when attempting to identify amebae in such cases. Our findings suggest that the leptomyxid ameba, regarded generally as having no pathogenic potential, can be the etiologic agent of fatal meningoencephalitis in humans as well as animals. We believe that the ameba described here belongs to a new genus and a new species. Further studies are in progress to determine the taxonomic position of this ameba within the order Leptomyxida.

LITERATURE CITED

1. **Anderson, M. P., J. E. Osterhuis, S. Kennedy, and K. Benirschke.** 1986. Pneumonia and meningoencephalitis due to amoeba in a lowland gorilla. J. Zoo Anim. Med. **17:**87–91.
2. **Anzil, A. P., C. Rao, M. A. Wrzolek, J. H. Sher, and P. B. Kozlowski.** 1989. Acanthamebic meningoencephalitis in an AIDS patient—an autopsy report. J. Neuropathol. Exp. Neurol. **48:**313.
3. **Bhagwandeen, S. B., R. F. Carter, K. G. Naik, and D. Levitt.** 1975. A case of *hartmannellid* amebic meningoencephalitis in Zambia. Am. J. Clin. Pathol. **63:**483–492.
4. **Carter, R. F., G. J. Cullity, V. J. Ojeda, P. Silberstein, and E. Willaert.** 1981. A fatal case of meningoencephalitis due to a free-living ameba of uncertain identity-probably *Acanthamoeba* sp. Pathology **13:**51–68.
5. **Clelend, P. G., R. V. Lawande, G. Onyemelukwe, and H. C. Whittle.** 1982. Chronic amebic meningoencephalitis. Arch. Neurol. **39:**56–76.
6. **Cox, E. C.** 1980. Amoebic meningoencephalitis caused by *Acanthamoeba* species in a four month old child. J. S.C. Med. Assoc. **76:**459–462.
7. **De Jonckheere, J. F.** 1987. Epidemiology, p. 127–147. *In* E. G. Rondanelli (ed.), Amphizoic amoebae: human pathology. Piccin Nuova Libraria, Pavia, Italy.
8. **Duma, R. J., W. B. Helwig, and A. J. Martinez.** 1978. Meningoencephalitis and brain abscess due to a free-living amoeba. Ann. Intern. Med. **88:**468–473.
9. **Hoffmann, E. O., C. Garcia, J. Lunseth, P. McGarry, and J. Coover.** 1978. A case of primary amebic meningoencephalitis. Light and electron microscope and immunohistologic studies. Am. J. Trop. Med. Hyg. **27:**29–38.
10. **Kwame Ofori-Kwakye, S., D. G. Sidebottom, J. Herbert, E. G. Fischer, and G. S. Visvesvara.** 1986. Granulomatous brain tumor caused by *Acanthamoeba*. J. Neurosurg. **64:**505–509.
11. **Lalitha, M. K., V. Anandi, A. Srivastava, K. Thomas, A. M. Cherian, and S. M. Chandi.** 1985. Isolation of *Acanthamoeba culbertsoni* from a patient with meningitis. J. Clin. Microbiol. **21:**666–667.
12. **Martinez, A. J.** 1980. Is *Acanthamoeba* encephalitis an opportunistic infection? Neurology **30:**567–574.
13. **Martinez, A. J.** 1982. Acanthamoebiasis and immunosuppression. J. Neuropathol. Exp. Neurol. **41:**548–557.
14. **Martinez, A. J.** 1985. Free-living amebas: natural history, prevention, diagnosis, pathology, and treatment of disease. CRC Press, Boca Raton, Fla.
15. **Martinez, A. J., C. Sotelo-Avila, H. Alcala, and E. Willaert.** 1980. Granulomatous encephalitis, intracranial arteritis and mycotic aneurism due to a free-living ameba. Acta Neuropathol. **49:**7–12.
16. **Matson, D. O., E. Rouah, D. Lee, R. T. Armstrong, J. T. Parke, and C. J. Baker.** 1988. Acanthamoeba meningoencephalitis masquerading as neurocysticercosis. Pediatr. Infect. Dis. J. **7:**121–124.
17. **Page, F. C.** 1988. A new key to freshwater and soil gymnamoebae. Fresh Water Biological Association, Ambleside, Cumbria, United Kingdom.
18. **Robert, V. B., and L. B. Rorke.** 1973. Primary amebic encephalitis probably from *Acanthamoeba*. Ann. Intern. Med. **79:**174–179.
19. **Rodriguez, J. Y., M. L. G. Chavez, R. J. E. Olivera, and A. J.**

Martinez. 1989. Encefalitis por une nueva amiba de vida libre, probablemente leptomyxid. Patologia (Mexico City) **27:**137–141.

20. **Rutherfoord, G. S.** 1986. Amoebic meningo-encephalitis due to a free-living amoeba. S. Afr. Med. J. **69:**52–55.

21. **Seidel, J. S., P. Harmatz, G. S. Visvesvara, A. Cohen, J. Edwards, and J. Turner.** 1982. Successful treatment of primary amebic meningoencephalitis. N. Engl. J. Med. **306:**346–348.

22. **Singh, B. N., and V. Hanumaiah.** 1979. Studies on pathogenic and non-pathogenic amoebae and the bearing of nuclear division and locomotive form and behaviour on the classification of the order Amoebida. Ind. J. Microbiol. Monogr. **1:**1–80.

23. **Stehr-Green, J. K., T. M. Bailey, and G. S. Visvesvara.** 1989. The epidemiology of *Acanthamoeba* keratitis in the United States. Am. J. Ophthalmol. **107:**331–336.

24. **Visvesvara, G. S.** 1987. Laboratory diagnosis, p. 193–215. *In* I. Rondanelli (ed.), Amphizoic amoebae. Piccin Nuova Libraria, Pavia, Italy.

25. **Visvesvara, G. S.** 1988. Acanthamoebiasis and naegleriosis, p. 723–730. *In* A. Balows, W. J. Hausler, Jr., M. Ohashi, and A.

Turano (ed.), Laboratory diagnosis of infectious diseases: principles and practice, vol. 1. Springer-Verlag, New York.

26. **Visvesvara, G. S., and W. Balamuth.** 1975. Comparative studies on related free-living and pathogenic amebae with special reference to *Acanthamoeba*. J. Protozool. **22:**245–256.

27. **Visvesvara, G. S., and C. S. Callaway.** 1974. Light and electron microscopic observations on the pathogenesis of *Naegleria fowleri* in mouse brain and tissue culture. J. Protozool. **21:**239–250.

28. **Visvesvara, G. S., S. S. Mirra, F. H. Brandt, D. M. Moss, H. M. Mathews, and A. J. Martinez.** 1983. Isolation of two strains of *Acanthamoeba castellanii* from human tissue and their pathogenicity and isoenzyme profiles. J. Clin. Microbiol. **18:**1405–1412.

28a.**Visvesvara, G. S., and J. K. Stehr-Green.** 1990. Epidemiology of free-living ameba infections. J. Protozool. **37:**25S–33S.

29. **Wessel, H. B., J. Hubbard, A. J. Martinez, and E. Willaert.** 1980. Granulomatous amebic encephalitis (GAE) with prolonged clinical course, C.T. scan findings, diagnosis by brain biopsy, and effect of treatment. Neurology **30:**442.